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Cytotoxic T cell function in fish

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Abstract

Fish possess immunoglobulins, major histocompatibility complex (MHC), T-cell receptors, and lymphocyte populations analogous to B and T cells and can evoke specific immune responses against a variety of antigens. However, T-cell subsets have yet to be demonstrated and the information on cell-mediated immunity is limited. Here we briefly review our recent studies on specific cell-mediated immunity, particularly on cytotoxic T-cell function employing isogeneic fish and cell lines. Analyses of the graft-versus host reaction (GVHR) and cell-mediated cytotoxicity (CMC) against allogeneic erythrocytes or cell lines show alloantigen-specific cytotoxicity in clonal ginbuna crucian carp. We also describe specific cytotoxicity against virus-infected cells using clonal ginbuna and a syngeneic cell line. Lastly, we report MHC-restriction in CMC against virus-infected cells using homozygous clonal rainbow trout and trout cell line which share the same MHC class I allele. These studies on CMC strongly suggest the presence of antigen specific cytotoxic T cells in teleosts and functional similarities between the immune systems of fish and higher vertebrates. Experimental model systems established in these studies can be applied to the investigation of protective antigens to induce cell-mediated immunity for the development of fish vaccines.

Keywords: Cell-mediated immunity; Cytotoxic T cells; MHC genes; MHC class I; Ginbuna; Rainbow trout

1. Introduction

Teleost fish can evoke a variety of specific immune functions, i.e. antibody production to a wide range of antigens, acute allograft rejection, delayed hypersensitivity reaction (DTH), mixed leukocyte reaction (MLR) and in vitro antibody production where T cells, B cells and macrophages co-operate in a similar manner to that of mammals (reviewed in Ref. [1]). More recently major histocompatibility complex (MHC) and T-cell receptor (TCR) genes, molecules that are involved in specific recognition of antigens, have been isolated from teleosts and elasmobranchs (review in Refs. [2,3] for MHC and Ref. [4] for TCR). However, T-cell subsets have yet to be demonstrated and information on specific cell-mediated immunity (i.e. the function of cytotoxic T cells, CTL) is relatively scarce when compared to those on humoral immunity due to the lack of

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Abbreviations: AS: a fibroblastic line derived from Atlantic salmon viscera; CCV: channel catfish virus; CHNV: crucian carp haematopoietic necrosis virus; CTL: cytotoxic T lymphocyte; EVA: eel virus from America; IHNV: infectious haematopoietic necrosis virus; IPNV: infectious pancreatic necrosis virus; NCC: nonspecific cytotoxic cells; PBL: peripheral blood leukocytes; RTG-2: a fibroblastic line derived from rainbow trout gonad.

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in vivo and in vitro model systems together with appropriate molecular and cellular markers.

It is well known that cell-mediated killing is a most important defence mechanism in the control of viral disease. In mammals, natural killer (NK) cells play an important role as the first line of defence against viral infection and antigen-specific $CD8^+$ T-lymphocytes play an essential role to lyse virus-infected cells and protect against re-infection [5]. However, nothing is known about CTL-mediated virus-specific cytotoxicity in fish due to the lack of suitable experimental systems.

Viral diseases are a serious problem in both farmed freshwater and marine fishes and cause heavy economic losses for the fish farming industry throughout the world [6]. There are no effective measures to control viral diseases for which antibiotics are not applicable. Vaccination is an alternative to control fish disease and many attempts have been made to develop viral vaccines including subunit recombinant vaccine as well as conventional inactivated or attenuated vaccines (reviewed in Ref. [7]). However, vaccination against viral disease is limited and only a few vaccines are available at present, e.g. recombinant infectious pancreatic necrosis virus (IPNV) vaccine for salmon in Norway, iridovirus vaccine for red sea bream in Japan and grass carp haemorrhage disease vaccine in China (reviewed in Ref. [7]). Vaccines for infectious haemotopoietic necrosis virus (IHNV) and viral hemorrhagic septicaemia virus (VHSV), which are the most important viral diseases of trout and salmon, have not yet been successful beyond the experimental stage. More recently, DNA vaccines have been shown to be effective in protecting rainbow trout against lethal infection of IHNV [8] and VHSV [9]. Development of such vaccines will require identification and characterization of viral antigens which induce protective immunity. Most studies have focused on protective antigens or epitopes neutralized by antibodies ([10] for IHNV; [11] for VHSV) and not those involved in cell-mediated immunity due to the lack of suitable assay systems. Cell-mediated immunity is potentially effective against intracelluar viruses and therefore protective antigens or epitopes should be analysed in this light.

Here we briefly review our recent studies on in vivo and in vitro allo- and virus-specific cell-mediated cytotoxicity (CMC) in clonal ginbuna with the syngeneic cell lines and MHC-restricted CMC against virus-infected cells in rainbow trout. We also describe the possibilities for the use of assay systems to analyse the protective antigens or epitopes involved in cell-mediated immunity for the development of fish vaccines as well as to investigate the recognition and killing mechanisms of specific cell-mediated immunity.

2. In vivo studies on specific cell-mediated immunity in fish

Most information on in vivo cell-mediated immunity has been obtained from transplantation experiments, particularly graft rejection (host versus graft rejection) studies (reviewed in Ref. [2]). First set allografted skin and/or scale is rejected in an acute manner in teleosts. Accelerated response on second-set grafts is commonly observed in all groups of fish. These features suggest that teleosts possess specific cell-mediated immunity, as characterized by antigen specificity and immunological memory.

Graft-versus-host reaction (GVHR) is also a representative phenomenon of cell-mediated immunity, in which $CD4^+$ and $CD8^+$ T-lymphocytes play a major role. Recently we demonstrated the presence of GVHR in a teleost fish employing a model system of clonal triploid ginbuna (*Carassius auratus langsdorfii*) and tetraploid ginbuna-goldfish (*Carassius auratus*) hybrids [12]. When triploid cells, sensitized by scale grafts from tetraploid donors, were injected into tetraploid recipients, a typical GVHR was induced, leading to death of the recipients within 1 month. A wide variety of organs and tissues showed pathological changes during the course of the clinically apparent graft-versus-host disease (GVHD) including enlargement of the spleen, infiltration of mononuclear cells and focal necrosis particularly in the skin, liver and lymphoid tissues. Ploidy analyses revealed that donor cells greatly increased in the host liver and spleen, and recipient cells as well as donor cells proliferated in the spleen and head-kidney.

Ginbuna crucian carp and goldfish are different at the subspecies level, although goldfish are believed to be produced from crucian carp thousands of years ago in China. Therefore, triploid ginbuna donor cells may not recognize antigens originating from goldfish in

Table 1
In vitro specific cell-mediated cytotoxicity in fish

Effector cells	Target cells	Requirement of sensitization	Species	Characteristics of cytotoxicity and cells involved in killing [References]
Kidney leukocytes	TNP-modified autologous spleen cells	In vitro culture with target cells	Carp	Lysis of not allogeneic, but autologous hapten-modified cells [14]
PBL	Long-term catfish cell lines	No	Channel catfish	Cytocytotoxicity against allogeneic, but not xenogeneic targets [17]
PBL	Long-term catfish cell lines	In vitro culture with target cells in one-way MLC	Channel catfish	Lack of allospecificity, NK cells [18]
PBL and kidney leukocytes	Allogeneic erythrocytes	Two in vivo immunizations	Ginbuna crucian carp and ginbuna-goldfish hybrid	Allospecific killing and no cytotoxicity against autologous or isogeneic targets [21]
Head-kidney and spleen cells	Allogeneic cell lines	At least one in vivo immunization	Ginbuna crucian carp and ginbuna-goldfish hybrid	Allospecific killing and no cytotoxicity against syngeneic cell line [22]

tetraploid recipients as allogenetically different targets and therefore the killing mechanism may be different from those in GVHR reported in mammals and birds. In order to clarify the above problem we tried to demonstrate GVHR in an additional teleost model system consisting of clonal diploid and triploid amago salmon (*Oncorhynchus rhodurus*). The homozygous clonal amago salmon were produced by artificial gynogenesis suppressing the first mitosis in the first generation followed by suppression of Meiosis II in the second generation. In this amago salmon system, triploid grafts on clonal diploid hosts evoked an acute rejection within 12 days, while diploid grafts on triploid hosts, and grafts among clonal diploid amago salmon were accepted. The clinical signs of GVHD were observed in the recipients 1 week after donor cell injection as a loss of appetite and appearance of solid faeces, followed by haemorrhage, local swelling of the ventral skin and an enlargement of the spleen [13]. Water temperature and frequency of sensitization appeared to be important to induce GVHR in amago salmon and donors kept at 20°C had to be sensitized three times in advance by grafting scales of the proposed host. This suggests that recognition and/or effector functions are specific events. Ploidy analyses also showed a great increase of donor cells in most of the lymphoid tissues. Most features of acute GVHD in amago salmon are quite similar to those found in the ginbuna and ginbuna-goldfish hybrid system, and therefore suggest the existence of similar mechanisms in the ginbuna and the amago system.

3. In vitro specific CMC against allogeneic cells

In vitro CMC has been documented for carp (specific CMC against hapten-modified autologous cells [14]) and channel catfish (non-specific cytotoxic cells [15]; PBL-derived cytotoxic cells against allogeneic and virus-infected target cells [16]; PBL-derived cytotoxic cells against long-term cultured allogeneic cells [17]; mixed leukocyte culture (MLC)-generated cytotoxic cells [18]; Table 1). However, cytotoxicity against allogeneic cells in channel catfish was not allo-specific and the effector cells resemble NK cells more than cytotoxic T cells (see review [19,20]). Moreover, it should be noted that

all effector cell populations of catfish mentioned above are cytotoxic against allogeneic targets even without sensitization.

We have recently established in vitro CMC assays for ginbuna using fresh erythrocytes as target cells and demonstrated specific killing of allogeneic erythrocytes [21]. Lymphocyte enriched fractions from fish sensitized in vivo by erythrocyte injection or scale grafting, efficiently lysed allogeneic erythrocytes, but did not lyse isogeneic or autologous erythrocytes. We have also developed in vitro CMC assays using allogeneic cell lines (CFS, CFK and CFO-2) established from three different clonal ginbuna as target cells and demonstrated specific killing of allogeneic cell lines [22]. Specific CMC was induced by intravenous immunization with allogeneic cell lines, but not the syngeneic cell line.

The characteristics of GVHR and in vitro CMC obtained with ginbuna and amago salmon are as follows: (a) repeated sensitization of effector cell donors was required to induce cytotoxicity; (b) effector cells killed allogeneic recipients or cells, but not isogeneic targets or syngeneic cells; and (c) the possible involvement of antibody-dependent CMC (ADCC) was excluded because the activity of effector cells of non-sensitized ginbuna was not detected by the addition of immune plasma. These results strongly suggest that cytotoxicity in ginbuna is mediated by allo-reactive cytotoxic T lymphocytes (CTL) rather than non-specific cytotoxic cells or NK cells, and the number of CTL is greatly increased by in vivo sensitization as reported in the cloning of catfish cytotoxic cells in which prior immunization and MLC markedly enhanced resulting in the increase of cloning efficiency [23].

4. Specific-CMC against a virus-infected syngeneic cell line

Specific recognition and killing mechanisms of CMC against virus-infected syngeneic cells have been well elucidated in mammals. In brief, $CD8^+$ CTL recognize MHC class I molecules/viral antigenic peptide complexes on the surface of infected cells through the TCR and kill these infected cells in direct cell to cell contact [24]. Until recently, nothing was known about CTL-mediated virus-specific cytotoxicity in fish due to the lack of suitable experimental systems, although a few papers have described the lysis of virus-infected cells by NK-like cells in rainbow trout [25,26] and in channel catfish [16].

We recently demonstrated virus-specific CMC employing clonal ginbuna (S3n clone) and a syngeneic cell line (CFS) established from S3n clone together with two infectious viruses: IPNV and eel virus from America (EVA). Peripheral blood lymphocytes (including thrombocytes) from S3n ginbuna sensitized with IPNV-infected CFS cells lysed the virus-infected CFS cells (immunogen) more efficiently than CFS cells infected with a different virus, EVA (non-immunogen) [27]. To induce cytotoxicity the fish had to be sensitized at least twice. In addition, effector cells from fish sensitized with IPNV-infected syngeneic cells did not lyse IPNV-infected xenogeneic cells (carp cell line: EPC and rainbow trout cell line: RTG-2). Although this study showed virus-specificity of the CMC, requirement of genetic identity between effector and target cells for the CMC remains to be investigated because allogeneic target cells were not used.

Further study was carried out using crucian carp haematopoietic necrosis virus (CHNV) which has been recently isolated from ginbuna and showed high mortality in ginbuna when i.p. injected. Cytotoxic activity of effector cells from S3n ginbuna against CHNV-infected syngeneic cells (CFS cells) was observed after primary infection with a sublethal dose of CHNV and secondary infection enhanced the cytotoxicity [28]. This cytotoxicity was not induced against either virus-infected allogeneic cells (CFK cells) or different virus (EVA)-infected syngeneic cells. The virus titres from infected fish reduced as the cytotoxic activity increased, suggesting that specific cell-mediated immunity in fish plays an important role in the defence against virus infection. These results showed that the specific CMC against virus-infected cells was restricted to certain allotypes, in agreement with CTL activity in higher vertebrates. The next question to be answered was if this restriction involves differences in MHC class I allele as in higher vertebrates. MHC class I loci have not been elucidated yet in ginbuna, and therefore another system had to be established (Table 2).

Table 2
In vitro cell-mediated cytotoxicity against virus-infected cells in fish

Effector cells	Target cells	Requirement of sensitization	Species	Characteristics of cytotoxicity and cells involved in killing [References]
Kidney and spleen cells, PBL	IPNV-infected, RTG-2 and AS	No	Atlantic salmon, rainbow trout	Higher killing activity against virus-infected cells than uninfected cells [25]
Head-kidney leukocytes	IPNV-infected RTG-2	No	Rainbow trout	Involvement of several cell types in the killing of virus-infected allogeneic target [26]
PBL	Long-term catfish cell lines and CCV-infected allogeneic and autologous cells	No	Channel catfish	Two populations of PBL-derived, cytotoxic effectors: one lyses allogeneic cells and other lyses virus-infected targets, probably not CTL and ADCC, but NK-like cells [16]
PBL (lymphocyte and thrombocytes)	IPNV- and EVA-infected syngeneic cell line	At least two in vivo immunizations	Ginbuna crucian carp	Virus specific killing, lysis of virus-infected syngeneic cells, but not xenogeneic cells [27]
PBL (lymphocyte and thrombocytes)	CHNV-infected syngeneic cell line	At least two in vivo immunizations	Ginbuna crucian carp	Virus specific killing, no cytotoxicity against virus-infected allogeneic cells and different virus-infected syngeneic cells [28]
PBL (lymphocyte and thrombocytes)	IHNV-infected RTG-2 (MHC class I matched with effector cells)	DNA immunization with IHNV-G	Rainbow trout	MHC class I restricted cytotoxicity, CTL [35]

AS, a fibroblastic line derived from Atlantic salmon viscera; CCV, channel catfish virus; CHNV, crucian carp haematopoietic necrosis virus; CTL, cytotoxic T lymphocyte; EVA, eel virus from America; IHNV, infectious haematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; NCC, nonspecific aptotoxic cells; PBL, peripheral blood leukocytes; RTG-2, a fibroblastic line derived from rainbow trout gonad.

5. MHC class I restricted cytotoxicity against virus-infected cells in rainbow trout

The MHC encodes two classes (I and II) of structurally and functionally distinct glycoproteins that present antigenic peptides to T cells and thus initiate specific immune responses. In mammals classical MHC class I (class Ia) are polymorphic molecules that mainly present peptides derived from intracellular proteins to $CD8^+$ cytotoxic T cells [24] and therefore play an essential role in cell-mediated immunity. To date, MHC genes including class I*A*, *B2m*, class II*A* and class II*B* have been reported from more than 25 species of teleosts and elasmobranchs [3,29] and extensive sequence variability was detected in several species of teleost (reviewed in Ref. [2]). Despite sequence analysis of variable MHC class I genes in fish, no information on polymorphism of fish MHC class I genes based on true alleles had been available until extensive allelic polymorphism of MHC class I genes based on defined loci was demonstrated in shark [30]. Furthermore, neither the function nor the distribution of molecules had been described for the fish MHC genes, although some MHC I genes in cartilaginous and bony fishes showed features resembling mammalian classical MHC class I by sequence conservation, polymorphic nature and ubiquitous tissue expression. Very recently we obtained the first indication that fish MHC molecules might be involved in allo-antigen recognition as mammalian MHC molecules. We found a strong correlation between MHC I sequences and intensity of skin allograft rejection in shark [31]. Namely, allografts between littermate sharks with the same MHC I allele survived for 2 months, while allograft rejection between different MHC I alleles occurred within 1 month. These results suggest that MHC I molecules encoded by MHC I genes isolated by Okamura et al. [30], or molecules encoded by genes co-segregated with the MHC I, are recognized as alloantigens and therefore are functional. Recently we also found a polymorphic rainbow trout MHC class Ia locus. The degree of variability at this locus was unexpectedly high, and it seems to be the single dominant classical MHC class I locus. Despite intensive investigations we could not find the second classical MHC I locus, although others claim that there might be two loci for MHC Ia in rainbow trout [32]. The locus seems to be involved in immune responses, as we found up-regulation of mRNA expression of the MHC Ia locus as well as that of β2m after IHNV infection of RTG-2 cells [33]. This stimulation upon infection and co-regulation with β2m agrees well with findings for classical MHC I in mammals. We also obtained a monoclonal antibody (MAb) against a recombinant protein of trout MHC Ia allele, *Onmy UBA**501 and found by immunohistochemistry that the MHC I molecule is predominantly distributed in leukocytes, endothelia and epithelia, resembling the distribution pattern of MHC Ia in higher vertebrates [34]. All the data above mentioned indicate that trout and shark MHC I genes highly resemble classical MHC I genes in mammals and suggest the similar function of fish MHC Ia molecules in antigen presentation as in mammals.

In vivo and in vitro allo- and virus-specific cytotoxicity described here suggest genetically restricted target cell recognition. However, no direct evidence of the genetic restriction has been provided to date. We extended our research to look at the MHC restriction in CMC against virally infected cells. Cytotoxic T cells taken from an individual recovering from a viral infection are capable of killing only virally infected cells which share an MHC I allele with the host. Accordingly, we should use effector and target cells with the same MHC I allele to study the MHC-restricted, antigen-specific CTL response in fish. We used homozygous clonal rainbow trout which have been produced by the same method as for amago salmon mentioned above. After extensive analysis of trout MHC Ia genes we found that one of the clones, C25 shares the same allele, Onmy-UBA*501 with the rainbow trout cell line, RTG-2. C25 fish and fish with a different allele were DNA-immunized with a plasmid encoding glycoprotein G of IHNV. Peripheral blood leukocytes (PBL) were isolated and used as effector cells against IHNV infected or uninfected cells in a non-radioactive cytotoxicity assay. PBL from DNA immunized C25 fish efficiently killed infected MHC I-matched RTG-2 cells, while the same PBL did not kill either uninfected RTG-2 cells nor infected or uninfected MHC I-mismatched RTE cells [35]. PBL from non-immunized C25 fish and from immunized or non-immunized fish with a different allele did not lyse the target cells. These results suggest that specific CMC against virus-infected cells is an MHC class I restricted event.

6. Future directions

Assay systems for fish immune responses have only been available for humoral immunity such as antibody response and nonspecific immunity such as phagocytic and killing activities of phagocytes, complement and lysozyme activities. As reviewed in this paper, however, several in vivo and in vitro assay systems measuring specific CTL activity have become available in fish. These assay systems can be used for the evaluation of vaccine potency, effects of immunomodulators such as, immunosuppressors, immunostimulants, adjuvants, etc. as has been evaluated from the perspective of humoral immunity or nonspecific immunity. In particular, these assays should be used for the development of fish vaccines, design of which is a matter of trial and error at present, analysing the protective antigens or epitopes involved in cell-mediated immunity.

6.1. Analysis of protective antigens and/or epitopes involved in cell-mediated immunity

Among fish rhabdoviruses, MAbs which mostly recognize the neutralizing epitopes on the viral glycoprotein G have been developed for IHNV [10] and VHSV [11]. These studies indicated that the G protein is the most important component of IHNV and VHSV as a protective antigen. In order to identify protective epitopes, neutralizing activity of the MAbs in vitro was first determined and their in vivo protective ability was investigated by passive immunization followed by challenge with virus [11,36]. However, no attempts have been made so far to analyze epitopes involved in cell-mediated immunity due to the lack of suitable assay systems. For this analysis, the donor has to be sensitized by live attenuated or DNA vaccines rather than by killed vaccines to effectively induce cell-mediated immunity. In particular, one major benefit of DNA vaccine is the endogenous synthesis of the encoded protein, allowing presentation of the foreign antigen by MHC class I resulting in CTL induction. One possible strategy for the analysis is as follows: effector cell donors can be sensitized by DNA immunization with plasmid DNA encoding viral proteins (i.e. mutant sequences of viral glycoprotein (G) genes of IHNV under the control of a cytomegalovirus promoter). Lymphocytes are isolated from effector donors and cocultured with IHNV infected target cells or transfected cells expressing viral proteins on the cell surface using assay systems described in this paper. In this system lymphocytes isolated from donors sensitized by DNAs expressing the G protein which lacks the epitope involved in cell-mediated immunity would not kill target cells.

6.2. Studies on recognition and killing mechanisms in specific cell-mediated immunity

The present review clearly demonstrates the presence of allo- and virus-specific cytotoxic T cells in teleosts from the functional point of view. However, precise mechanisms of recognition and killing, or the nature of the cells involved in cell-mediated immunity is not known. A number of mechanisms may be involved to recognize and kill allogeneic and/or virus-infected cells in vivo and in vitro. It may be possible to answer these questions using suitable reagents or cell lines, i.e. monoclonal antibodies (MAbs) against T-cell markers, molecular probes for fish TCR and MHC genes, and clones of T cells.

There is no doubt that identification and separation of T cells and/or T-cell subsets is crucial for further study of specific cell-mediated immunity. However, T cells have not been identified and attempts to produce MAbs against T-cell markers have met with difficulty (reviewed in Ref. [37]). Recently, a MAb recognizing antigenic determinants on thymocytes and peripheral T-lymphocytes of sea bass, *Dicentrarchus labrax* has been produced [38]. By using the MAb, localization of T-lymphocytes involved in allograft rejection has been assessed and a high density of immunopositive lymphocytes (T cells) was found in allografts [39]. Such MAbs against T-lymphocytes are surely useful to obtain pure populations to analyze the function of T-cell subsets and the mechanism of CMC. The necessity of leukocyte-derived cell lines is also apparent since much information at the cellular level has been obtained using long term cell lines in channel catfish [40]. Recently, greatly enhanced cytotoxicity after MLC of catfish PBL with X-irradiated allotargets was found [18] and various clonal alloantigen-dependent cytotoxic cell lines have been established from MLC [23]. It

is obvious that great progress should be made in the field of fish immunology if such cell lines and MAbs against leukocytes of homozygous clonal fish were available. At present, however, each research groups are working with different tools and species of fish, e.g. channel catfish, sea bass, ginbuna crucian carp, rainbow trout, etc. which show great diversity observed in the immune system.

In mammals, cytotoxicity of CTL is largely restricted to MHC class Ia molecules, whereas regulatory interactions between leukocytes are restricted to MHC class II molecules. It of interest to know if this functional division between MHC class I and II already exists at the level of fish. The class restriction of mixed leukocyte response (MLR) and antigen-specific T-cell response could be assessed by employing MAbs against MHC class I and II, antigen specific T-cell clones along with clonal fish. These studies should provide great insight into the evolution of MHC function.

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